15-Lipoxygenase-1 Production is Lost in Pancreatic Cancer and Overexpression of the Gene Inhibits Tumor Cell Growth[1]

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Abstract

Pancreatic cancer patients have an abysmal prognosis because of late diagnosis and lack of therapeutic options. Pancreatic intraepithelial neoplasias (PanINs), the precursor lesions, are a potential target for chemoprevention. Targeting eicosanoid pathways is an obvious choice because 5-lipoxygenase (5-LOX) has been suggested as a tumor promoter in pancreatic carcinogenesis. Here we provide evidence that 15-lipoxygenase-1 (15-LOX-1) expression and activity may exert antitumorigenic effects in pancreatic cancer. Reverse transcription–polymerase chain reaction (RT-PCR) and Western blot analysis showed absence or very weak expression of 15-LOX-1 in all pancreatic cancer cell lines tested. 15-LOX-1 was strongly stained in normal ductal cells, tubular complexes, and centroacinar cells, but no staining was seen in islets, cancer cells, PanIN lesions, or in tumor cells in lymph node metastases, indicating that 15-LOX-1 expression is lost during tumor development in human pancreas. Overexpression of 15-LOX-1 in pancreatic tumor cells or treatment with its arachidonic acid–derived metabolite resulted in decreased cell growth. These findings provide evidence that loss of 15-LOX-1 may play an important role in pancreatic carcinogenesis, possibly as a tumor suppressor gene. Thus, induction of 15-LOX-1 expression may be an attractive option for the prevention and treatment of pancreatic cancer.

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Introduction

In the last decade, death rate from all cancers combined has decreased by 1.5% per year among men and by 0.8% per year among women, whereas the mortality rate has decreased from lung, colorectal, prostate, and breast cancer, but not pancreatic cancer [1]. In the US, 33,730 new cases as well as 32,300 deaths from pancreatic cancer are estimated in 2006 [1]. Pancreatic cancer is the fourth leading cause of cancer-related death in the US and ranks third in men between 40 and 59 years of age [1]. This devastating cancer is one of the cancer sites for which survival has not improved substantially over the past 25 years [2]. The overall 5-year relative survival rate has only increased from 3% to 5% within the last three decades [1]. Incidence and mortality rates are equal between men and women and have not declined since 1930 [1]. Indeed, incidence has increased in Japanese and African Americans over recent decades [3–5]. Pancreatic cancer has an abysmal prognosis because of late diagnosis and lack of therapeutic options. Only 9% to 20% of patients proceed to surgical resection and even with surgery; these patients often have poor long-term survival [6]. Many patients have recurrent disease by 12 months after surgery [6]. The 2- and 5-year survival times are at about 40% and 20%, respectively, and the median survival time is 19 months when patients receive adjuvant chemotherapy [7,8]. Thus, new targets need to be identified for early diagnosis, prevention, and treatment of this disastrous disease [9]. Pancreatic intraepithelial neoplasias (PanINs) are the histologically defined precursor lesions of pancreatic cancer in the small ducts and ductules. PanINs are an ideal target for chemoprevention or early treatment [10–12].

The results from epidemiological and animal studies suggest that a high fat consumption is associated with an increased incidence and growth of tumors at several specific organ sites

Abbreviations: 15-LOX, 15-lipoxygenase-1; ANOVA, analysis of variance; DMEM, Dulbecco's modified Eagle's medium; PanIN, pancreatic intraepithelial neoplasia; RT-PCR, reverse transcription–polymerase chain reaction

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including pancreas, colon, breast, and prostate [13–16]. In these tissues, it has been demonstrated that linoleic acid promotes carcinogenesis [17].

Recent reviews have highlighted the importance of lipoxygenase pathways in fat metabolism and in the regulation of pancreatic cancer cell proliferation and survival [18,19]. Lipoxygenases (LOXs) are lipid-peroxidizing enzymes that are categorized according to their position in the oxygenation of arachidonic acid, e.g. 15-lipoxygenase (15-LOX) oxygenates arachidonic acid at C-15 [20]. 15-Lipoxygenase-1 (15-LOX-1) metabolizes not only linoleic acid, the predominant polyunsaturated fatty acid in the human diet, to 13(*S*)-hydroxyoctadecadienoic acid (13(*S*)-HODE) but also arachidonic acid to 15(*S*)-hydroxyeicosatetraenoic acid (15(*S*)-HETE) and it is expressed in reticulocytes, eosinophils, macrophages, and the airway epithelium [20,21]. Numerous clinical studies on LOX expression in human tumors as well as in animal models indicate different roles of the distinct LOX isoforms in carcinogenesis [22,23]. In fact, different LOX isoforms exhibit either protumorigenic or antitumorigenic activities and modulate the tumor response in a tissue-specific manner. Data suggest that the 5-LOX and platelet-type 12-LOX exhibit procarcinogenic activities, whereas 15-LOX-1 and 15-LOX-2 may suppress carcinogenesis.

In pancreatic carcinogenesis, 5-LOX has been suggested to be a new tumor promoter, because it is upregulated in pancreatic cancer cells, in all grades of human PanINs, and in early lesions of pancreatic cancer in two different animal models (EL-Kras mice and *N*-nitroso-bis(2-oxopropyl)amine-treated hamsters) [24–26].

In contrast, 15-LOX-1 is downregulated in human esophageal, gastric, colorectal, and breast carcinomas and nonsteroidal antiinflammatory drugs can induce apoptosis in these cancer cells independently of cyclooxygenase-2 (COX-2) inhibition [17,27–31]. Furthermore, downregulation of 15-LOX-1 seems to be an early event in the adenoma to carcinoma sequence of the colon [30]. In the past, conflicting data on 15-LOX-1 expression in colorectal carcinomas were published, since one group described elevated levels of 15-LOX-1 in human colon tumors [20]. However, the current working hypothesis is that expression of 15-LOX-1 exerts an antitumorigenic effect in the colon but is protumorigenic in the prostate [32–34]. In addition, 15-LOX-1 has been suggested as a tumor suppressor gene, because low micromolar concentrations of either 5-aza-2-deoxycytidine (5-Azadc) [a potent methyltransferase inhibitor (MTI)] or suberoylanilide hydroxamic acid (a histone deacetylase inhibitor) induce the expression of 15-LOX-1 in human colorectal cancer cells, causing growth arrest, differentiation, and/or apoptosis [35].

To date, nothing is known about the role of 15-LOX-1 in pancreatic cancer. Therefore, in the present study, we investigated the expression of 15-LOX-1 in normal and pathologic pancreatic tissues and the effect of 15-LOX-1 expression on pancreatic cancer cell proliferation. We were curious whether or not 15-LOX-1 could serve as a new tumor suppressor and whether or not it can be used as a potent therapeutic target in the fight against pancreatic cancer.

Materials

RPMI 1640, Dulbecco's modified Eagle's medium (DMEM), DMEM/Ham's F12, penicillin–streptomycin solution, and trypsin–EDTA solution were purchased from Gibco (Invitrogen, Karlsruhe, Germany). FBS was from PAA (PAA Laboratories, Cölbe, Germany). The 15-LOX-1 antibody was a generous gift from Dr. Mary Mulkins (Roche Bioscience, Palo Alto, CA). 15(*S*)-HETE was purchased from Cayman (Cayman Chemicals, Ann Arbor, MI).

Cell Lines and Cell Culture

The cell lines used, i.e., PANC-1, Capan I, Capan II, MiaPaCa2, AsPC-1, and S2-O13, were established from patients with pancreatic adenocarcinoma. All of the human pancreatic cancer cell lines, except S2-O13, were purchased from the American Type Culture Collection (ATCC, Rockville, MA). S2-O13 cells were provided by Dr. M. A. Hollingsworth (Eppley Cancer Institute, Omaha, NE). A normal pancreatic epithelial cell line (AMA) from bovine origin was kindly provided by Dr. R. Jesenofski and Prof. M. Löhr (Deutschen Krebsforschungszentrum, Heidelberg, Germany).

S2-O13 and MiaPaCa2 were cultured in DMEM. Capan I, Capan II, AsPC-1, and PANC-1 were grown in RPMI 1640. AMA cells were cultivated in DMEM/Ham's F12.

Media were supplemented with 10% FBS and 1% penicillin–streptomycin and cells were grown as monolayers in a humidified atmosphere of 5% CO_2 at 37°C. Adherent cells were detached by trypsinization with 1 ml trypsin–EDTA solution (Gibco, Invitrogen).

Stable Transfection

The plasmids of pcDNA3.1 and pcDNA3.1–15-LOX-1 containing the coding region of human 15-LOX-1 were transfected into MiaPaCa2 and S2-O13 cells according to the manufacturer's protocol using a reagent (Lipofectamine 2000; Invitrogen). After 24 hours, fresh medium was added to the cells containing the selection reagent G418 (100 μg/ml) (Sigma-Aldrich, Munich, Germany). Selection was continued for 1 week, with the medium being refreshed every alternate day. After 1 week, cells were maintained at 20 μg/ml G418.

Clones were isolated and 15-LOX-1 overexpression was confirmed by Western blot and activity assay analyses.

Reverse Transcription–Polymerase Chain Reaction (RT-PCR) for 15-LOX-1

Total RNA from Su8686, PANC-1, MiaPaCa2, BxPc3, AsPC-1, Capan I, Capan II, Colo357, T3M4, S2-O13, and AMA cells as well as from human monocytes was isolated according to the manufacturer's protocol using a purification kit (GenElute Mammalian Total RNA Kit; Sigma-Aldrich, Munich, Germany). To ensure purity, the isolated RNA was treated with DNAse I (Sigma-Aldrich, Munich, Germany). The final reaction volume was 15 μl, containing 5 μg of RNA, 0.5 μl of DNAse I, 1.5 μl of PCR buffer, and water. Before adding 2.5 μl of 10 mM EDTA, the mix was incubated at 37°C for 15 minutes. Subsequently, the mix was

incubated at 65°C for 20 minutes and then cooled down for 5 minutes at 4°C.

Cellular RNA was reverse-transcribed into cDNA according to the manufacturer's protocol (GeneAmp RNA PCR Core Kit; Applied Biosystems, Foster City, CA). The total reaction volume was 20 μl containing the following: 1 μg of total RNA, 25 mM $MgCl_2$, 2 μl of 10× PCR buffer II, 2 μl of deoxyribonucleotide triphosphates (10 mM), 1 μl of RNase inhibitor (20 U/μl), 1 μl of murine leukemia virus reverse transcriptase (50 U/μl), 1 μl oligo dT primer and RNase/DNase–free water. The thermocycler protocol for the RT phase is one cycle at 20°C for 10 minutes, 42°C for 15 minutes, and one cycle at 99°C for 5 minutes.

After the RT reaction, 1 μl of the cDNA obtained from RT-PCR was used for 15-LOX-1-specific PCR. Primers were designed based on the sequence of human or bovine 15-LOX-1, respectively (Table 1).

The PCR profile was one cycle at 94°C for 5 minutes, 94°C for 1.5 minutes, 56°C for 1.5 minutes, and 36 cycles at 72°C for 2.5 minutes. The same PCR profile was used to verify bovine 15-LOX-1. As a control, β-actin PCR was performed using human and bovine primer sequences (Table 1). The final PCR product was separated on 1% agarose gel containing ethidium bromide.

Western Blot Analysis for 15-LOX-1

Western blot analysis was carried out to determine the specificity of the 15-LOX-1 antibody, the expression of 15-LOX-1 in tumor cell lines, and to confirm 15-LOX-1 overexpression in transfected MiaPaCa2 and S2-O13 cells.

Cells seeded onto 100-mm dishes were lysed (50 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, and 1 mM aprotinin) and sonicated, using ultrasound three times for 10 seconds. The protein content of lysates was measured using bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL).

Proteins in 10 μg of each pancreatic cancer cell lysate were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE 4–12%) (NU-Page; Invitrogen). Proteins were transferred to nitrocellulose membranes (BioRad, Hercules, CA) by electroblotting using a transfer blotting apparatus (NU-Page; Invitrogen).

Nitrocellulose membranes were subsequently blocked in Tris-buffered saline (TBS) containing 5% nonfat milk and 0.1% Tween-20, then treated with 15-LOX-1 antiserum at 1:10,000 dilution in 5% nonfat milk, TBS containing 0.1% Tween-20 overnight at 4°C. After washing, the membrane was incubated with horseradish peroxidase (HRP)–conjugated goat anti–rabbit antibody at 1:2000 dilution for 1 hour. 15-LOX-1 protein was detected by chemiluminescence treatment (Amersham, Piscataway, NJ) and capture of light emission on an X-ray film (Fujifilms, Düsseldorf, Germany).

LOX Activity Assays

LOX activity was quantified by measuring HETE formation in cell-free protein extracts. The amount 250 μg of cytosolic protein homogenates were incubated with 100 μM arachidonic acid for 15 minutes at 37°C. The incubations were terminated by the addition of 40 μl of 1 M sodium formate buffer (pH 3.1) and products were extracted by with a modification of the Bligh and Dyer procedure as described [36]. Products were analyzed by reverse-phase HPLC on a 5-μm YMC-Pack ODS-AM column (25 × 0.46 cm^2, YMC Europe, Schermbeck, Germany) with a 1-cm guard column using the solvent system of methanol/water/acetic acid (82:18:0.01 by volume) and a flow rate of 0.5 ml/min. Elution was monitored at 235 nm with a diode array detector (Bio-Tek Kontron 540; Kontron Instruments, Neufahrn, Germany). The products were identified by comparing retention times and peak areas with those of authentic external 15(*S*)-HETE standard (Reatec, Weiterstadt, Germany). The retention time for 15(*S*)-HETE was 28.0 minutes.

Treatment with 15(S)-HETE

To examine the responsiveness of pancreatic cancer cells to 15(*S*)-HETE, MiaPaCa2 and S2-O13 cells were treated with 0.5 and 1 μM 15(*S*)-HETE for 24 to 120 hours and cell proliferation was measured by cell counting using a *Neubauer* chamber and Guava PC (Guava Technologies, Inc., Hayward, CA).

At the beginning, 50,000 cells were seeded into 60-mm dishes. After 24 hours, the medium was replaced by medium containing 15(*S*)-HETE (2 ml/dish). Medium was changed again 72 hours after induction. As a negative control, cells were treated with the solvent (ethanol).

Cell Proliferation Analysis

The proliferation of 15-LOX-1–expressing MiaPaCa2 and S2-013 cells was determined by cell counting using a *Neubauer* chamber and Guava PC (Guava Technologies).

MiaPaCa2–15-LOX-1 and S2-O13–15-LOX-1 stably transfected and MiaPaCa2–Mock– and S2-O13–Mock–transfected cells were seeded at 2.5 × 10^4/ml into 60-mm culture dishes. Each dish contained 5 × 10^4 cells at the starting point. Triplicates were counted every 24 hours up to 120 hours. This experiment was repeated three times.

Additionally, cell proliferation was measured using the WST-1 assay from Roche (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. A total

Table 1. 15-LOX-1 Primer Sequences.

	5′ Primer	3′ Primer
15-LOX-1 (human)	5′–CATCTATCGGTATGTGGA–3′	5–GAAGTTGGGCAGTGTC–3′
15-LOX-1 (bovine)	5′–CGGTGGCTCAGTGGTAAAG–3′	5′–ACCATGAGGTGCTTCCGTCT–3′
Actin (human)	5′–CTTCCTGGGCATGGAGTCCT–3′	5′–CCGCCGATCCACACAGAGTA–3′
Actin (bovine)	5′–CGGCATTCACGAAACTACCTT–3′	5′–TAGAAGCATTTGCGGTGGA–3′

of 1000 cells of each cell line, i.e., MiaPaCa2–15-LOX-1, MiaPaCa2–Mock, S2-O13–15-LOX-1 and S2-O13–Mock, were plated into six wells of a 96-well plate using a 100-μl per well cell culture volume. A total of 10 μl of the ready-to-use WST-1 reagent was added to the cells and incubated for 1 hour at 37°C under 5% CO_2 atmosphere. As a negative control, cell culture medium alone was used. The absorbance of the samples was measured against the background using a microplate reader at a wavelength of 450 nm.

Measurement of Apoptosis

MiaPaCa2–15-LOX-1 and S2-O13–15-LOX-1 stably transfected cells were seeded at 2.5×10^4/ml into 60-mm culture dishes. Each dish contained 5×10^4 cells at the starting point. Annexin V binding (Nexin Assay; Guava Technologies) was performed according to the manufacturer's protocol. About 120 hours after seeding, cells were trypsinized and centrifuged at 350*g* for 10 minutes at 4°C. The supernatant was removed and the pellet was washed with 1 ml of cold Nexin buffer. The supernatant was removed and the pellet was resuspended in 1 ml of cold Nexin buffer.

The cell number was adjusted to 200,000 cells/ml. The amounts 5 μl of Annexin V–PE and 5 μl of 7-AAd were added to 40 μl of cell suspension in 1× Nexin buffer, shortly vortexed, and then the reaction was incubated shielded from light, on ice for 20 minutes. A total of 450 μl of cold Nexin buffer was added to each reaction tube, vortexed, and then acquired on the Guava PC (Guava Technologies).

Immunohistochemistry for 15-LOX-1

Eleven surgical pancreatic specimens were obtained from patients with chronic pancreatitis and twelve were from pancreatic adenocarcinoma. Twelve pancreas specimens from multiorgan donors were included as controls. In addition, nine lymph node metastases and five liver metastases from patients with pancreatic cancer were included. The Human Subjects Committee at the University of Heidelberg, Germany, approved this study.

All specimens were fixed in 10% buffered formalin, paraffin-embedded, and processed for histology using conventional methods. Sections (4 μm thick) were prepared from the paraffin blocks. After deparaffinization, the slides were submerged in methanol containing 0.3% hydrogen peroxide for 30 minutes at room temperature to inhibit endogenous peroxidase activity. Thereafter, slides were washed in Tris-buffered saline (TBS; 0.1 M, pH 7.4). The slides were incubated with normal goat serum for 30 minutes at room temperature and then with the primary antibody directed against 15-LOX-1 (rabbit polyclonal, diluted 1:2500 in TBS containing 1% BSA) for 18 hours at 4°C. The slides were then washed again in TBS and incubated with biotinylated secondary anti–rabbit antibody (Multilink; Bio Genex, San Ramon, CA) for 10 minutes at 37°C. Detection of the antibody complex was performed by the streptavidin–peroxidase reaction kit using DAB as chromogen. To ensure the specificity of the primary antibody, control tissue sections were incubated in the absence of primary antibody. Counterstaining was performed with Hematoxylin Gil No. 2 (Sigma-Aldrich, St. Louis, MO). The stained tissue samples were verified by two pathologists.

Statistical Analysis

Data on 15-LOX-1 expression by immunohistochemistry in humans were analyzed by analysis of variance (ANOVA) with the Student-Newman-Keuls post hoc test for multiple comparisons. Paired *t*-test and Friedman's repeated-measures ANOVA on ranks (with Tukey's post hoc test for pairwise multiple comparison procedures) were used to analyze cell proliferation (WST-1 and cell counting).

Results

15-LOX-1 Expression in Pancreatic Tissues

15-LOX-1 expression in normal and neoplastic pancreas was analyzed by immunohistochemistry. The staining pattern indicated strong expression of 15-LOX-1 in normal ductal cells in normal as well as in diseased pancreas. However, larger ducts, e.g., the main pancreatic duct, did not stain for 15-LOX-1 in most biopsies. Furthermore, tubular complexes in normal, diseased, or neoplastic tissues showed marked expression of 15-LOX-1. However, tubular complexes were stained in only 6/12 normal pancreatic tissues but in all tissues obtained from patients with chronic pancreatitis (11/11) and pancreatic adenocarcinoma (12/12). In addition, centroacinar cells were positive for 15-LOX-1 whereas other acinar cells did not stain. Whenever 15-LOX-1 was expressed, the staining was specific and granular in the cytoplasm. Interestingly, PanIN-1 lesions were positively stained for 15-LOX-1, but more advanced PanIN lesions were negative. No expression of 15-LOX-1 was demonstrated in islet or cancer cells. Clearly, inside the peripancreatic lymph node metastases (9/9), as well as liver metastases (5/5), macrophages, but not metastatic cancer cells, stained positive for 15-LOX-1 (Figure 1). Detailed information on histology and staining intensity is shown in Table 2.

Generation of Stable 15-LOX-1–Expressing Pancreatic Cancer Cell Lines

Expression of 15-LOX-1 in various human pancreatic cancer cell lines was analyzed by RT-PCR and Western blot analysis. 15-LOX-1 mRNA and protein expression was below the level of detection in all cancer lines tested including Su8686, PANC-1, MiaPaCa2, BxPc3, AsPC-1, Capan I, Capan II, Colo357, T3M4, and S2-O13 (Figure 2). Lysates from human monocytes and HEK293 cells transiently expressing 15-LOX-1 were used as positive controls. In contrast, strong 15-LOX-1 mRNA expression was detected in normal ductal cells of bovine (AMA) origin (data not shown). This immortalized bovine ductal cell line was used because a normal human ductal cell line is not available. The polyclonal antibody was specific for 15-LOX-1 and did not detect any of the other human lipoxygenases (data not shown). The data suggest that 15-LOX-1 expression is lost during tumor development.

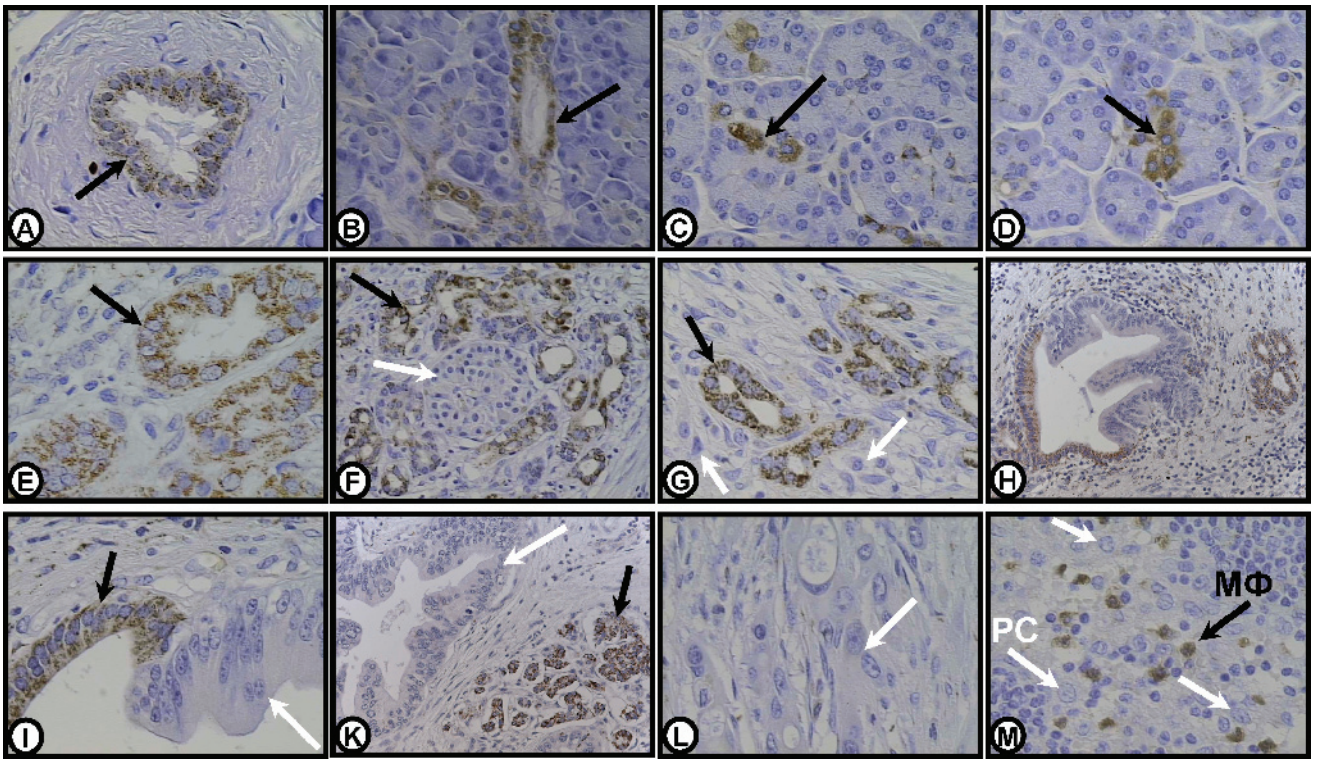

Figure 1. *Expression of 15-LOX-1 in human pancreatic tissues. Immunohistochemistry for 15-LOX-1 is shown in panels A to M. (A–D) Different normal human pancreas with stained ductal cells (A and B) and stained centroacinar cells (C and D) marked by black arrows; magnification, × 400. (E) Stained tubular complexes in chronic pancreatitis (black arrow); magnification, × 400. (F and G) Human pancreatic adenocarcinoma with intense positive staining in tubular complexes (black arrows) but unstained islet (white arrow) (F; magnification, × 200) and cancer cells (white arrow) (G; magnification, × 400). (H–L) Human pancreatic adenocarcinoma with intense positive staining in normal ductal cells (black arrow) and tubular complexes (black arrow) but unstained PanIN-2 (white arrow) (H and I) and PanIN-3 (white arrow) (K) lesions (H, I, and K; magnifications, × 200, × 400, and × 200, respectively) and cancer cells (white arrow) (L; magnification, × 400). (M) Positively stained lymphocytes (black arrow; MΦ = macrophage) and unstained cancer cells (white arrow; PC = pancreatic cancer cells) in a lymph node metastasis from pancreatic cancer; magnification, × 400. Immunohistochemistry for 15-LOX-1 used a rabbit polyclonal antibody, diluted 1:2500, and incubated overnight with DAB at 4°C. Panels were counterstained with hematoxylin.*

The eukaryotic expression vector pcDNA3 carrying the cDNA under the control of the cytomegalovirus promoter was used to stably express 15-LOX-1 in pancreatic cancer cells. Two cell clones, MiaPaCa2–15-LOX-1–cl13 and S2-O13–15-LOX-1–cl8, which showed strong expression of 15-LOX-1 by RT-PCR and Western blot analysis were selected for further experiments (Figure 3). The enzymatic activity of 15-LOX-1 in both cell lines was confirmed by the formation of 15(*S*)-HETE in protein lysates on incubation with arachidonic acid; whereas in mock-transfected clones expressing the vector construct, no 15(*S*)-HETE production was observed (Figure 4).

Effects of 15-LOX-1 Overexpression and 15-LOX-1 Metabolites on Tumor Cell Proliferation

When compared with mock-transfected cells, 15-LOX-1 overexpression significantly inhibited the proliferation of MiaPaCa2 and S2-O13 cells, as measured by both cell counting ($P < .001$) and WST-1 assay ($P = .0003$ and $P = .001$) (Figure 5, *A* and *B*). Highly significant differences ($P < .0001$) in the cell numbers were observed at 96 and 120 hours after seeding.

Treatment of the parental MiaPaCa2 and S2-O13 cells with the arachidonic acid–derived 15-LOX-1 product 15(*S*)-HETE caused growth inhibition by 17% at 0.5 μM (data not shown) and by 21% at 1 μM concentration ($P < .001$), with markedly reduced cell numbers at 96 and 120 hours ($P < .0001$) (Figure 6*A*). In contrast, addition of the linoleic acid product 13(*S*)-HODE did not affect cell proliferation at 1 μM but only at 50 μM and more (not shown). No additional growth-inhibitory effect was observed by exogenous 15(*S*)-HETE in 15-LOX-1–overexpressing cells MiaPaCa2–15-LOX-1 and S2-O13–15-LOX-1.

To understand the growth-inhibitory effect, we investigated apoptosis by performing annexin V binding and examined cell cycle distribution by FACS analysis. Overexpression of 15-LOX-1 in pancreatic cancer cells caused apoptosis (Figure 6*B*), whereas no change in cell cycle was observed (data not shown).

Discussion

At present, the best chance to improve on statistics for pancreatic cancer would be prevention or early detection before this cancer becomes invasive, because no effective treatment is available. Even small resectable tumors recur in most cases within 2 years after primary surgery or have metastasized, which becomes evident months or years later. Chemotherapy only prolongs life by a matter of weeks, maintaining the unchanged abysmal prognosis of pancreatic cancer.

Table 2. Immunohistochemistry for 15-LOX-1 in Human Pancreatic Tissues.

Sample	Normal Ducts	Tubular Complexes	PanIN	Cancer Cells	Islet Cells	Acini	Centroacinar Cells
Normal (CD)							
1	+	Absent	Absent	Absent	Negative	Negative	+
2	++	+++	Absent	Absent	Negative	Negative	+++
3	++	Absent	Absent	Absent	Negative	Negative	++
4	++	Absent	Absent	Absent	Negative	Negative	+++
5	+	++	Absent	Absent	Negative	Negative	++
6	++	Absent	Absent	Absent	Negative	Negative	+++
7	++	Absent	Absent	Absent	Negative	Negative	+
8	+++	++	Absent	Absent	Negative	Negative	++
9	++	++	Absent	Absent	Negative	Negative	++
10	(+)	Absent	Absent	Absent	Negative	Negative	++
11	++	++	Absent	Absent	Negative	Negative	+++
12	++	++	Absent	Absent	Negative	Negative	++
CP							
1	+	+	Negative	Absent	Negative	Negative	+
2	Negative	+++	Absent	Absent	Negative	Negative	+
3	++	++	1a: ++; 2: negative	Absent	Negative	Absent	Absent
4	Negative	+	Negative	Absent	Negative	Negative	Negative
5	+++	+++	1a: +++	Absent	Negative	Negative	+++
6	++	++	Absent	Absent	Negative	Negative	++
7	+++	+++	1a: ++; 2: negative	Absent	Negative	+	+++
8	Negative	++	Absent	Absent	Negative	Negative	Negative
9	+++	+++	Absent	Absent	Negative	Negative	+
10	Negative	+++	Absent	Absent	Negative	Negative	+
11	+++	+++	1a/b: +++	Absent	Negative	Negative	+++
PC							
1	+++	+++	Negative	Negative	Negative	Negative	Absent
2	+++	+++	Negative	Negative	Negative	Negative	Absent
3	+++	+++	Negative	Negative	Negative	Negative	Absent
4	Absent	+++	Negative	Negative	Negative	Negative	Absent
5	+++	+++	Negative	Negative	Negative	Negative	Absent
6	+++	+++	1b: +	Negative	Negative	Negative	Absent
7	+++	+++	Negative	Negative	Negative	Negative	++
8	++	++	Negative	Negative	Negative	Absent	Absent
9	++	+++	Negative	Negative	Negative	Negative	++
10	+++	+++	Negative	Negative	Negative	Negative	+++
11	++	++	Negative	Negative	Negative	Negative	Absent
12	+++	+++	Negative	Negative	Negative	Negative	Absent
ANOVA with Student-Newman-Keuls	$P = .011$	$P < .001$	$P = .012$		No difference	No difference	$P = .003$
Normal *vs* chronic pancreatitis	$P = .445$	$P = .014$	$P = .017$*				$P = .068$
Normal *vs* pancreatic cancer	$P = .022$	$P < .001$	$P = .786$				$P = .002$
Chronic pancreatitis *vs* pancreatic cancer	$P = .011$	$P = .064$	$P = .013$†				$P = .076$

Because of the multiple groups, statistical analysis in the human samples was carried out by ANOVA with the Student-Newman-Keuls post hoc test for multiple comparisons.
CD, cadaver donor; *CP*, chronic pancreatitis; *PC*, pancreatic cancer; *PanIN*, pancreatic intraepithelial neoplasia, grades 1a, 1b, 2 and 3.
Absent: described structures are not present in this tissue section.
Negative: unstained but present structures.
+: weak positive staining.
++: positive staining.
+++: strong positive staining.
(+): very weak positive staining.
*No PanIN in CD.
†PanIN-2 and -3 are negative.

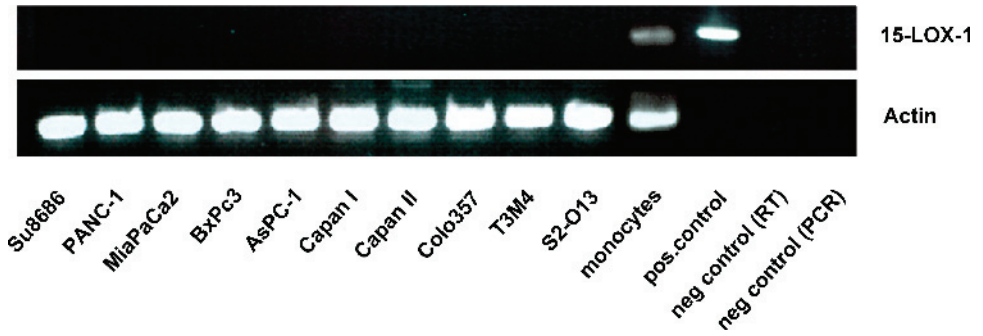

Figure 2. *Lack of 15-LOX-1 mRNA expression in pancreatic cancer cell lines compared to human monocytes (positive control). Total RNA was isolated, reverse-transcribed, and then amplified by PCR. The PCR product was separated on 1% agarose gel and visualized by ethidium bromide staining.*

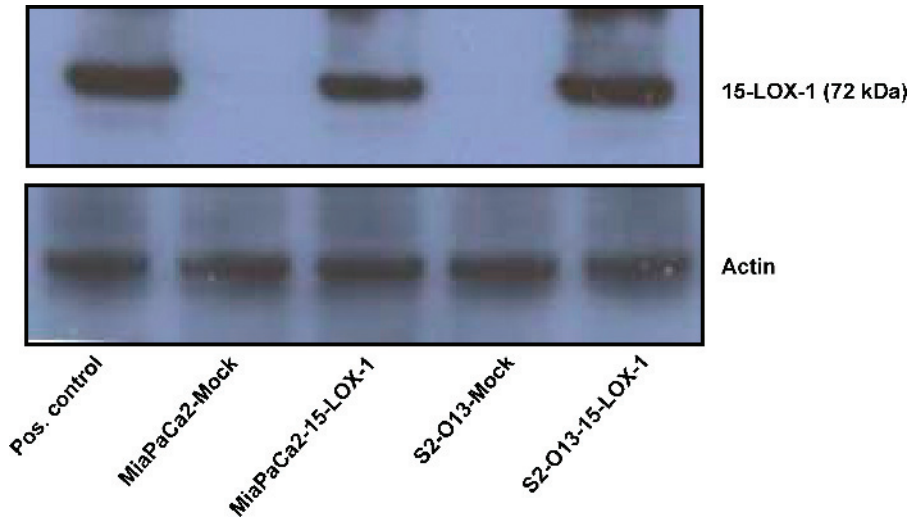

Figure 3. *Western blot analysis shows the expression of 15-LOX-1 protein in MiaPaCa2 and S2-O13 cells transfected with pcDNA3.1 (Mock) and pcDNA3.1–15-LOX-1. Protein lysates from 15-LOX-1–transfected HEK293 cells served as positive controls. Actin expression demonstrates equal loading.*

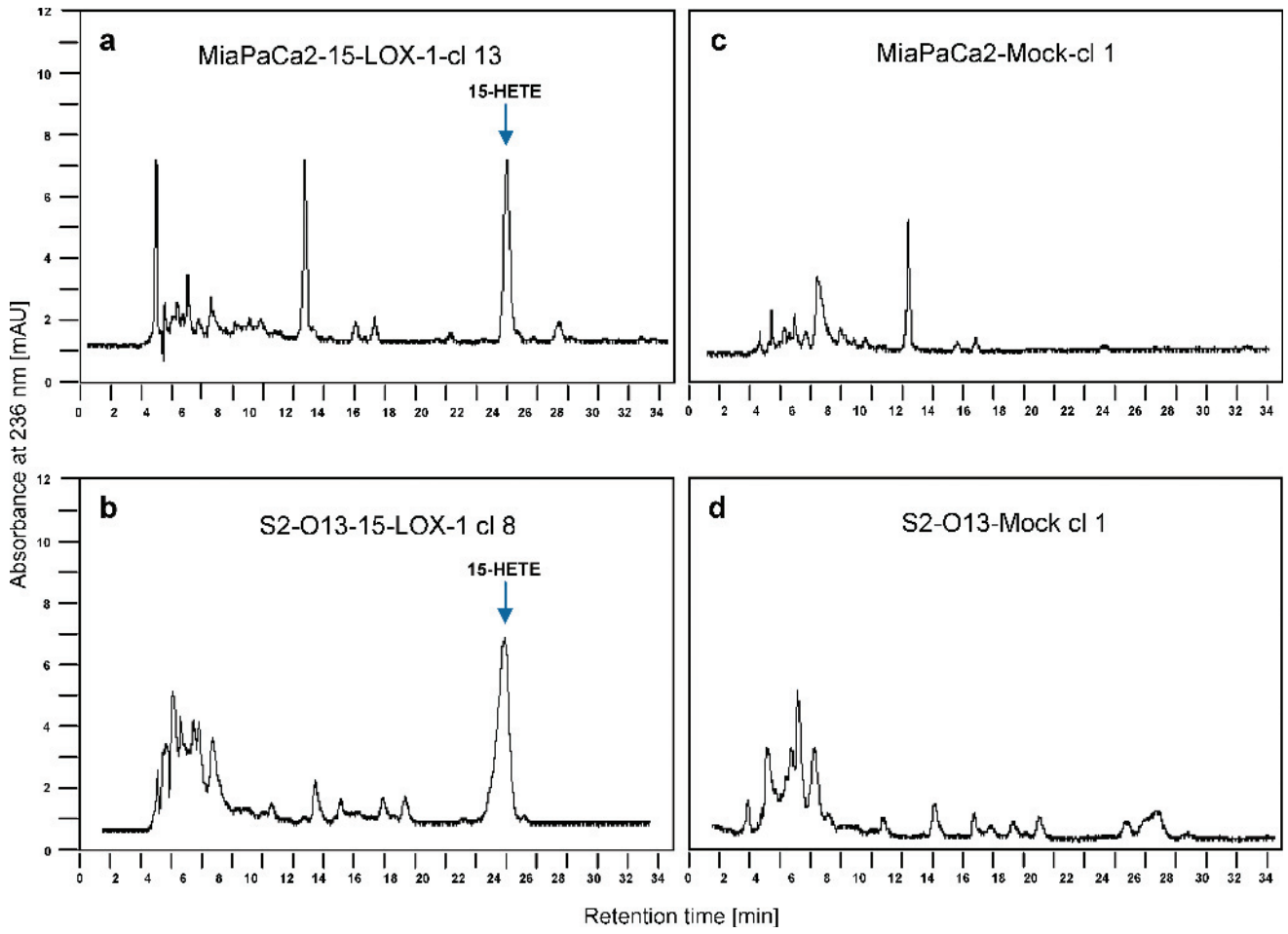

Figure 4. *15-LOX-1 activity in 15-LOX-1–expressing pancreatic cancer cells: reverse-phase HPLC analysis of products formed in cell extracts from stably transfected MiaPaCa2 and S2-O13 cells. Homogenates from cells were incubated in Tris EDTA buffer with 100 μM arachidonic acid for 15 minutes at 37°C. Products were extracted with methanol/dichloromethane (1:1 by vol.), dried under vacuum, redissolved in methanol/water/acetic acid (82:18:0.01, by vol.), injected on a 4-μm YMC-Pack ODS-H80 column, and eluted at 0.5 ml/min. The eluate was monitored at 235 nm. Authentic 15(S)-HETE was used as standard. The retention time of 15(S)-HETE was 28.0 minutes.*

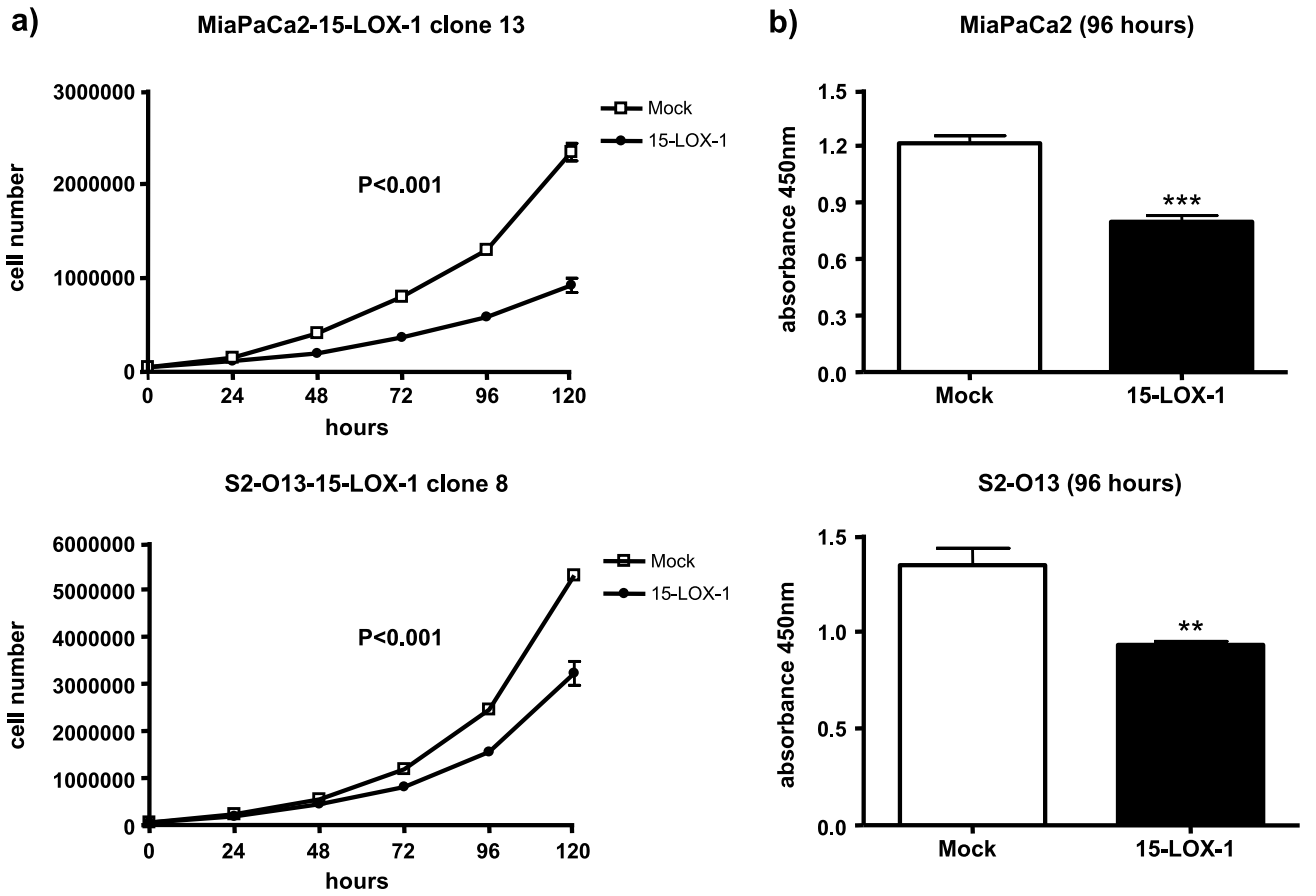

Figure 5. *15-LOX-1 inhibits cell proliferation in transfected MiaPaCa2 and S2-O13 cells shown by (A) cell counting and (B) WST-1 assay. **P ≤ .001; ***P ≤ .0001.*

The connection between inflammation and cancer goes back to Virchow in 1863 when he noted a *lymphoreticular infiltrate* in neoplastic tissues [37]. He suggested the origin of cancer in the context of chronic inflammation. Inflammatory cells and cytokines are more likely to contribute to tumor growth and progression than to mount the host antitumor response [37]. In addition, pancreatic cancer is characterized by a strong desmoplastic reaction and, in 1986, Dvorak showed that wound healing and tumor stroma formation share many important properties. He suggested tumors as wounds that do not heal [37]. The eicosanoid pathways appear to play an important role in tumor development, growth, and progression. For example, cyclooxygenases are the rate-limiting enzymes in prostaglandin synthesis and it is well accepted that the inducible isoform, COX-2, plays an important role in carcinogenesis [19,38]. Furthermore, intake of nonsteroidal antiinflammatory drugs reduces the risk of various cancers including colon cancer [39,40]. COX-2 and 5-LOX are expressed in pancreatic adenocarcinomas and PanINs from malignant and nonmalignant pancreatic tissues and, recently, 5-LOX has been suggested as a new tumor promoter in pancreatic carcinogenesis [25,26]. Six human lipoxygenases have been characterized to date. Whereas 5-LOX and 12-LOX obviously have rather protumorigenic effects on different tumor cells, the role of 15-LOX-1 is controversial [19]. Expression of 15-LOX-1 is associated with antitumorigenic effects in the colon but protumorigenic effects in the prostate, indicating tissue selectivity of LOX function [32,33]. In the present study, we have, for the first time, demonstrated expression of 15-LOX-1 in normal ductal cells, tubular complexes, PanIN-1 lesions and centroacinar cells, but loss of expression in PanIN-2 or PanIN-3 lesions and in cancer cells of different human pancreatic tissues. In addition, inflammatory cells in the stroma of pancreatic cancers were positively stained, as expected. Curious as to why normal epithelial cells in the pancreas but not malignant cells express this enzyme, we investigated the functional role of 15-LOX-1. In fact, 15(*S*)-HETE, the arachidonic acid–derived product of 15-LOX-1, exhibited growth-inhibitory effects. Interestingly, the linoleic acid–derived 13(*S*)-HODE, which has been shown to be the active 15-LOX-1 metabolite in colon and prostate cancer cells [28,33,41], had no growth modulatory effects in pancreatic cells when applied in concentrations below 50 μM (data not shown). Because none of the investigated pancreatic cancer cell lines express 15-LOX-1 at either the mRNA and the protein level, we stably transfected two pancreatic cancer cell lines (MiaPaCa2 and S2-O13) with 15-LOX-1 to restore expression of this enzyme. Significant growth inhibition and induction of apoptosis occurred after restoration of 15-LOX-1 activity. These results are in line with findings in colonic, gastric, and esophageal cancers where either 15-LOX-1 expression is downregulated in cancer cells and 13(*S*)-HODE or upregulation of 15-LOX-1 causes growth inhibition by inducing apoptosis [17,27–

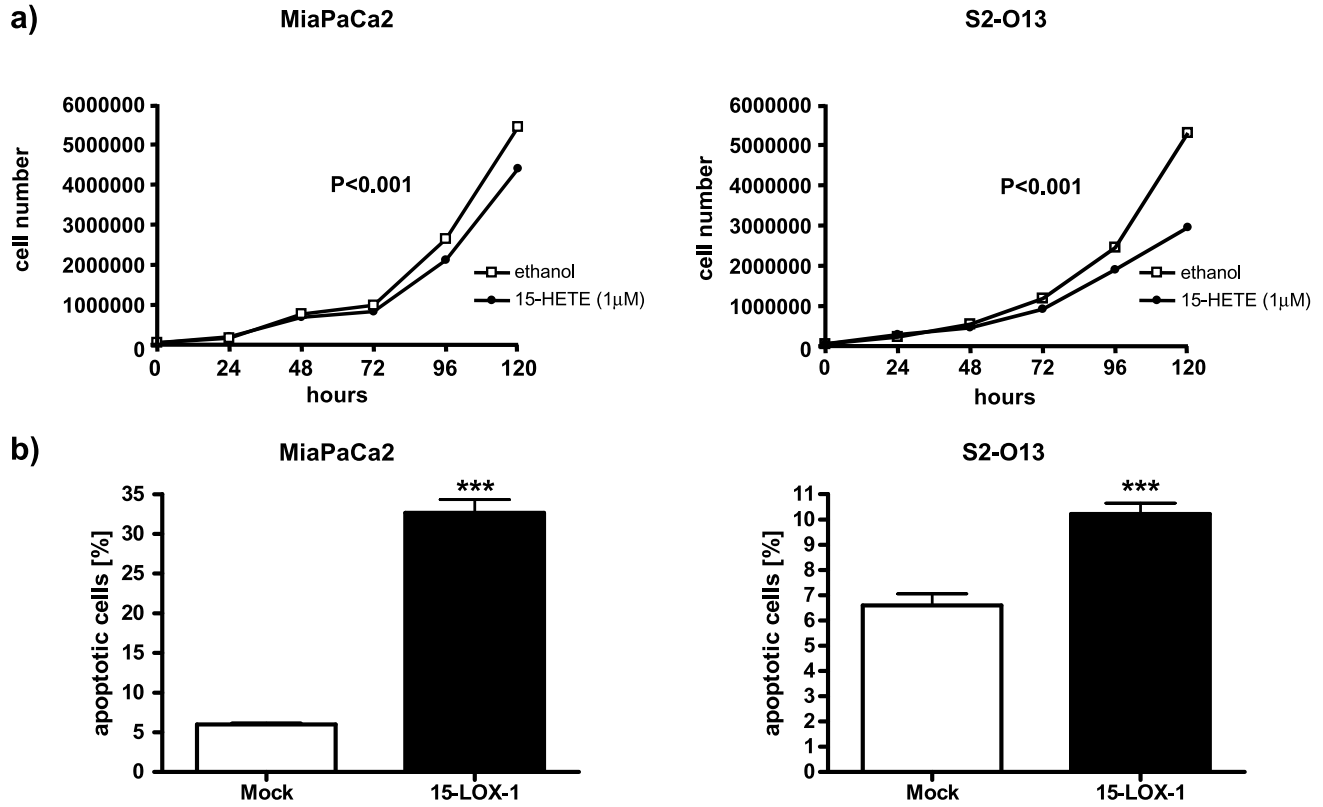

Figure 6. *(A) Treatment with 15(S)-HETE decreased cell proliferation of MiaPaCa2 (P < .001) and S2-O13 cells (P < .001) as shown by cell counting. (B) Overexpression of 15-LOX-1 in MiaPaCa2 and S2-O13 cells caused apoptosis. Significantly more apoptotic cells are present in 15-LOX-1 stably transfected cancer cells (***P < .0001). Apoptosis was measured after 120 hours using the Nexin assay from Guava.*

29,31]. Obviously, overexpression of the transcription factor GATA-6 in human colon cancer cells contributes to the multifactorial process of silencing 15-LOX-1 [42]. COX-2 inhibitors induce 15-LOX-1 expression through downregulation of GATA-6 [43]. An important role for the cyclic guanosine monophosphate/protein kinase G pathway in the restoration of 15-LOX-1 activity has been suggested [44]. In addition, IL-4 and IL-13 play important roles in transactivating 15-LOX-1, which can then induce p53 expression and phosphorylation/activation [32,45]. Other research groups found the 15-LOX-1 promoter to be exclusively methylated in cells incapable of expressing this enzyme, whereas 5-Azadc, a potent MTI, induces the expression of 15-LOX-1 in human colorectal cancer cells [35]. Reversing epigenetic silencing, using a MTI such as 5-Azadc, has become a new and important target approach for cancer prevention and therapy [35]. Another possible way to induce 15-LOX-1 expression is to use suberoylanilide hydroxamic acid, a histone deacetylase inhibitor, which also causes apoptosis in colorectal cancer cells [46].

It was recently shown that downregulation of 15-LOX-1 is an early event in the adenoma to carcinoma sequence in colonic cancer development [30,47]. We observed similar findings in pancreatic carcinogenesis, because 15-LOX-1 expression is lost in advanced PanINs as well as cancer cells, but not in PanIN-1 lesions. These findings are based on immunohistochemistry and need to be confirmed, e.g. by laser capture microdissection in future studies. It is widely accepted that PanIN-2 and -3 are precursor lesions for ductal adenocarcinoma, whereas PanIN-1 lesions are also found in many nonmalignant pancreatic tissues. Therefore, the switch to develop pancreatic adenocarcinoma may happen at PanIN-2 stage, when 15-LOX-1 is already downregulated.

These findings provide evidence that loss of 15-LOX-1 may play an important role early in pancreatic carcinogenesis, possibly as a tumor suppressor gene. Thus, 15-LOX-1 may be an attractive target for the prevention and treatment of pancreatic cancer.

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